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Award Number: DAMD17-03-1-0440

TITLE: Effect of HER-2/Neu Signaling on Sensitivity to TRAIL in Prostate Cancer

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REPORT DATE: June 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20041215 052

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 2003 – 31 May 2004)

4. TITLE AND SUBTITLE
Effect of HER-2/Neu Signaling on Sensitivity to TRAIL in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0440

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We have previously observed that low extracellular pH (pHe) promotes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity. In this study, we examined whether amiloride, which is an inhibitor of the Na^+/H^+ antiport and reduces intracellular pH (pHi), can augment TRAIL-induced apoptotic death. Human prostate adenocarcinoma DU-145 cells were treated with various concentration of TRAIL (10-200 ng/ml) and/or amiloride (0.1-1mM) for 4 h. Amiloride, which had little or no cytotoxicity, enhanced TRAIL-induced apoptosis. The TRAIL-mediated activation of caspase, and PARP cleavage, were promoted in the presence of amiloride. Western blot analysis showed that combined treatment with TRAIL and amiloride did not change the levels of TRAIL receptors (DR4, DR5, and DcR2) and antiapoptotic proteins (FLIP, IAP, and Bcl-2). However, unlike low extracellular pH, amiloride dephosphorylated Akt. Interestingly, amiloride also dephosphorylated PI3K and PDK-1 kinases along with PTEN and PP1α phosphatases. *In vitro* kinase assay revealed that amiloride inhibited phosphorylation of kinase as well as phosphatase by competing with ATP. Taken together, the present studies suggest that amiloride enhances TRAIL-induced cytotoxicity by inhibiting phosphorylation of the PI3K-Akt pathway-associated kinases and phosphatases.

14. SUBJECT TERMS
TRAIL apoptosis Akt PI3K PDK-1 PTEN

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

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Introduction :

The abnormalities of the tumor vasculature, i.e. a loss of the natural hierarchy of blood vessels, changes in the vascular density, and loss of the physiological regulation of blood perfusion, have been well documented (1). These abnormalities cause an insufficient blood supply and development of a pathophysiological tumor microenvironment including low extracellular pH (pHe) (2). Recently, we demonstrated that low pHe augments the effect of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a potent anticancer agent which induces apoptosis (3). The effect of a low pH is probably due to cytosol acidification and loss of the H^+ gradient which normally presents across the inner membrane of mitochondria during apoptosis. In this study, we investigated whether amiloride (3,5 diamino-6-chloro-N-(diaminomethylene) pyrazinecarboximide), an inhibitor of the Na^+/H^+ antiport, can promote TRAIL-induced apoptotic death. It is well known that mammalian cells possess effective intracellular pH (pHi) regulatory mechanisms, such as the Na^+/H^+ antiport and HCO_3^-/Cl^- exchange, through which the intracellular environment is maintained at a near neutral pH even in an acidic environment (4). We postulate that amiloride leads to acidification of the intracellular environment by blocking the pHi regulatory mechanisms to enhance TRAIL cytotoxicity.

TRAIL/APO-2L is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family. TRAIL is a 281-amino acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand (FasL) and TNF, the C-terminal extracellular region of TRAIL (amino acids 114-281) exhibits a homotrimeric subunit structure (5). TRAIL binds to death receptors such as TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and induces the apoptotic signal. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) which act as decoy receptors by inhibiting TRAIL signaling (6-12). Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif (9). The relative resistance of normal cells to the apoptotic inducing effects of TRAIL has been explained by the presence of large numbers of the decoy receptors in normal cells (13, 14). Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines (15) and melanoma cell lines (16). This discrepancy indicates that other factors such as death inhibitors including the FLICE-inhibitory protein (FLIP) (16), Fas-associated protein (FAP-1) (17), Bcl-2 (18), Bcl-X$_L$ (18), Bruton's tyrosine kinase (BTK) (19) silencer of death domain (SODD) (20), toso (21), inhibitor of apoptosis (IAP) (22), X-linked inhibitor of apoptosis (XIAP) (23), and survivin (24). Previous studies show that chemotherapeutic agents (15, 16, 25) and ionizing radiation (26) can increase TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP (16) or increasing DR5 gene expression in response to genotoxic stress (25, 26, 27). We originally hypothesized that amiloride promotes TRAIL-induced apoptotic death by modulating both the levels of TRAIL receptors and antiapoptotic molecules.

In addition to alterations of the levels of TRAIL receptors and antiapoptotic molecules, posttranslational modification of antiapoptotic molecules such as phosphorylation of Akt may play an important role in TRAIL sensitivity to the apoptotic-increasing effect of amiloride. Akt (also known as PKB) is a serine/threonine kinase. It was originally discovered as the cellular counterpart of the v-Akt transforming protein of a retrovirus (AKT8) that caused T-cell lymphomas in mice (28). Akt is recruited to the plasma membrane and activated by phosphorylation at threonine 308 and serine 473 in response to growth factors (29). The molecule responsible for the recruitment of Akt is phosphatidylinositol (PI) 3-kinase [PI(3)K] (29, 30). PI(3)K consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (P110) (31). PI(3)K phosphorylates phosphoinositides at the 3'-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (PIP_3) (32). PIP_3 facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology (PH) domain of Akt (32). At the plasma membrane, Akt is activated by phosphoinositide-dependent kinase-1 (PDK-1) through phosphorylation at threonine 308 and serine 473 (30, 33). Activated Akt is involved in phosphorylation of several transcription factors (e.g. NF-κB, Forkhead, CREB) (34, 35, 36) as well as proapoptotic molecules such as Bad and procaspase-9. Phosphorylation of Bad and procaspase-9 results in their inactivation and inhibits apoptosis (37, 38, 39). In this study, we observed that amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of Akt rather than by changing the levels of TRAIL receptors and antiapoptotic molecules.

Body :

Our grant proposal was initially designed to study the expression of human epidermal growth factor receptor-2 (HER-2)/neu and resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in prostate cancer cells. The planned research was to examine the PI3K-Akt signal transduction pathways that may be involved in TRAIL-induced apoptosis. As a first step we investigated whether modulation of the PI3K-Akt signals affects TRAIL-induced cytotoxicity. As DU-145 cells were treated with amiloride, an inhibitor of the PI3K-Akt signal, we observed that TRAIL-induced cytotoxicity was promoted. Our observations are illustrated below:

Amiloride enhances TRAIL-induced cytotoxicity

To investigate the effect of amiloride on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma DU-145 cells were treated with TRAIL in the presence or absence of amiloride. Figures 1A and 1B show that little or no cytotoxicity was observed with amiloride (0.1-1 mM) alone. In contrast, TRAIL-induced cytotoxicity, which was dependent upon concentration, was significantly increased in the presence of 1 mM amiloride (Fig. 1B). For example, when cells were treated for 4 h with 200 ng/ml TRAIL in the presence or absence of 1 mM amiloride, the survival fraction was 17% or 43%, respectively. Similar results were observed by DAPI staining (Fig. 1C). DAPI staining showed the presence of many cells with condensed nuclei, a characteristic morphological change in cells that is associated with apoptosis, when cells were treated TRAIL in combination with amiloride.
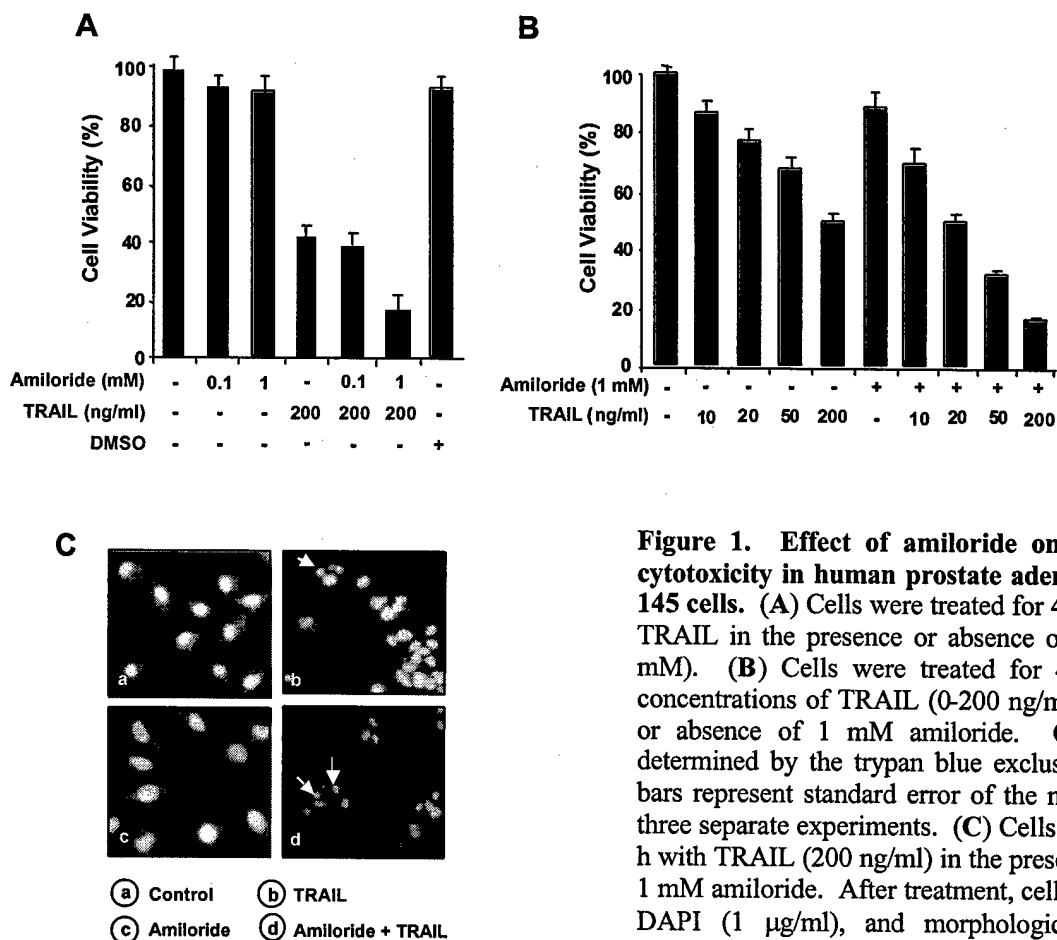
Figure 1. Effect of amiloride on TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells. (A) Cells were treated for 4 h with 200 ng/ml TRAIL in the presence or absence of amiloride (0.1-1 mM). (B) Cells were treated for 4 h with various concentrations of TRAIL (0-200 ng/ml) in the presence or absence of 1 mM amiloride. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. (C) Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, cell were stained with DAPI (1 µg/ml), and morphological features were analyzed with a fluorescence microscope. Nuclei of apoptotic cells are fragmented and condensed, indicated by arrows.

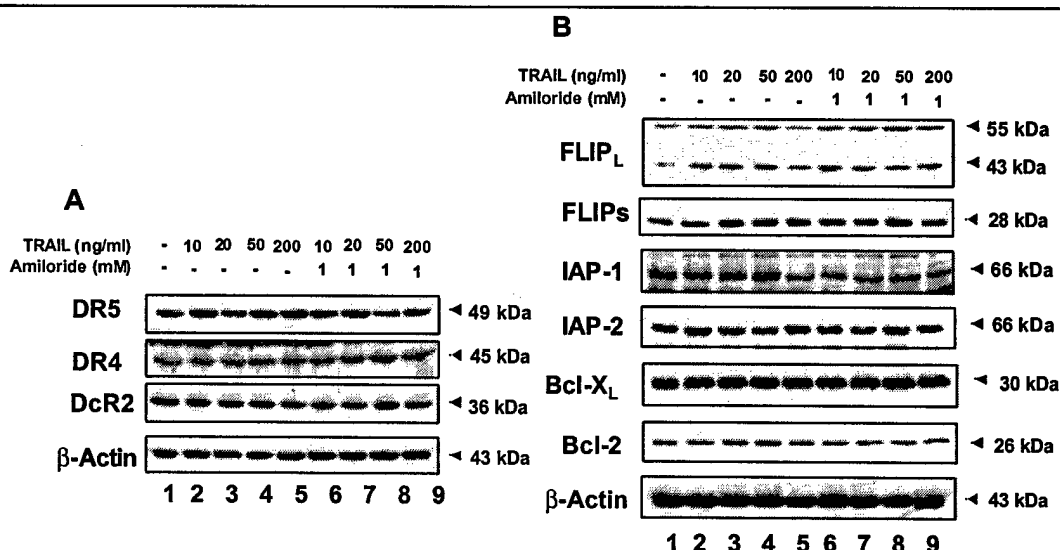## Effect of amiloride on TRAIL-induced apoptosis

Additional studies were designed to examine whether the combination of amiloride and TRAIL treatment enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis, in DU-145 cells. PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL (Fig. 2). This cleavage was enhanced by treatment with amiloride (Fig. 2). The cleavage of PARP was not observed by treatment with amiloride alone (data not shown). We extended our studies to investigate whether amiloride enhances TRAIL-induced cytotoxicity by increasing the activation of caspase. Figure 2 demonstrates that amiloride promoted TRAIL-induced caspase-8 activation. Western blot analysis shows that procaspase-8 (54/55 kDa) was cleaved to the intermediates (41 and 43 kDa) and active form (18 kDa) in the presence of TRAIL. The cleavage of procaspase-8 was promoted by treatment with amiloride. The combined treatment of TRAIL and amiloride also resulted in an increase in caspase-9 activation (Fig. 2). TRAIL induced proteolytic processing of procaspase-9 (48 kDa) into its active form (37 kDa). The activation of caspase-9 induced by TRAIL was enhanced in the presence of amiloride (Fig. 2). Amiloride also augmented TRAIL-induced caspase-3 activation (Fig. 2). Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) in the presence of TRAIL. The combined treatment with amiloride and TRAIL increased the level of the active form. Amiloride alone did not activate caspases (data not shown).
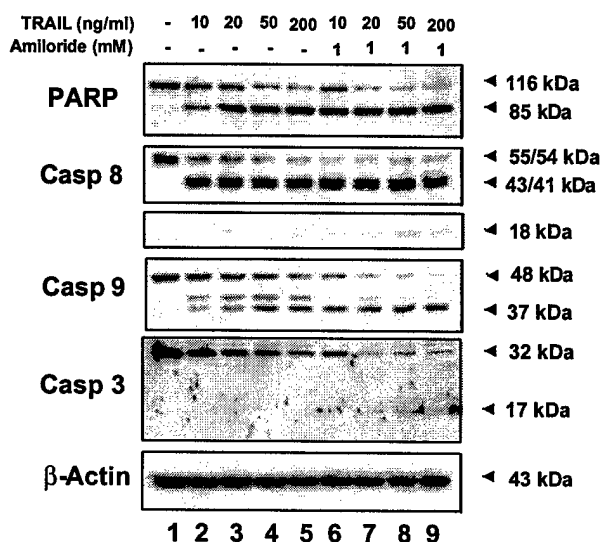
Figure 2. Effect of amiloride on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in DU-145 cells. Cells were treated for 4 h with various concentrations of TRAIL (0-200 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. Cell lysates were subjected to immunoblotting for caspase-8, caspase-9, caspase-3, or PARP. Antibody against caspase-8 detects inactive form (55/54 kDa), and cleaved intermediates (41, 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

Effect of amiloride on the level of TRAIL receptor family and antiapoptotic proteins

Previous studies demonstrate that increased DR5 by genotoxic agents (25, 26, 27) or decreased FLIP expression by glucose deprivation (42) is responsible for synergistic cytotoxicity with TRAIL. Thus, we examined whether changes in the amounts of TRAIL receptors and antiapoptotic proteins are associated with the promotion of apoptosis by TRAIL in combination with amiloride. DU-145 cells were treated with 200 ng/ml TRAIL in the presence of 1 mM amiloride. Data from western blot analysis reveal that the combined treatment did not signficantly alter the levels of DR4, DR5, DcR2, FLIP$_L$, FLIP$_S$, IAP-1, IAP-2, Bcl-X$_L$, and Bcl-2 (Fig. 3). Amiloride alone did not change the levels of TRAIL receptors and antiapoptotic proteins (data not shown).

6

B

| TRAIL (ng/ml) | - | 10 | 20 | 50 | 200 | 10 | 20 | 50 | 200 |
| Amiloride (mM) | - | - | - | - | - | 1 | 1 | 1 | 1 |

FLIP_L   ◄ 55 kDa
                                                                                                      ◄ 43 kDa

FLIPs   ◄ 28 kDa

IAP-1   ◄ 66 kDa

IAP-2   ◄ 66 kDa

Bcl-X_L   ◄ 30 kDa

Bcl-2   ◄ 26 kDa

β-Actin   ◄ 43 kDa

1 2 3 4 5 6 7 8 9

A

| TRAIL (ng/ml) | - | 10 | 20 | 50 | 200 | 10 | 20 | 50 | 200 |
| Amiloride (mM) | - | - | - | - | - | 1 | 1 | 1 | 1 |

DR5   ◄ 49 kDa

DR4   ◄ 45 kDa

DcR2   ◄ 36 kDa

β-Actin   ◄ 43 kDa

1 2 3 4 5 6 7 8 9

Figure 3. Intracellular levels of TRAIL receptors (A) or antiapoptotic proteins (B) during treatment with TRAIL in the presence or absence of amiloride. DU-145 cells were treated for 4 h with various concentrations of TRAIL (0-200 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. Actin was shown as an internal standard.

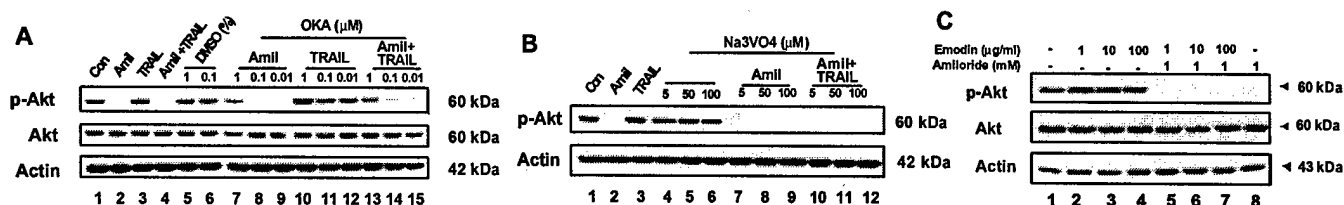## Effect of amiloride on Akt phosphorylation

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis (43). We postulated that amiloride inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity. To examine whether amiloride inhibits Akt activity by dephosphorylating Akt, DU-145 cells were treated with 1 mM amiloride for various periods (5-60 min) and the level of phosphorylated Akt was detected. Figure 4A shows that Akt was rapidly dephosphorylated within 5 min without changing the Akt protein level. TRAIL treatment did not alter amiloride-induced dephosphorylation of Akt (Fig. 4B). We further investigated whether low extracellular pH also dephosphorylates Akt. Figure 4C reveals that low extracellular pH did not alter the level of phosphorylated Akt. These results suggest that the mechansim of low extracellular pH-enhanced TRAIL cytotoxicity is not the same as that of amiloride-promoted TRAIL apoptotic death.

A

1 mM Amiloride

| | C | 5 | 10 | 20 | 30 | 60 | (min) |

p-Akt   60 kDa

Akt   60 kDa

Actin   43 kDa

1 2 3 4 5 6

B

| TRAIL (ng/ml) | - | 10 | 20 | 50 | 200 | 10 | 20 | 50 | 200 |
| Amiloride (mM) | - | - | - | - | - | 1 | 1 | 1 | 1 |

p-Akt   ◄ 60 kDa

Akt   ◄ 60 kDa

Actin   ◄ 43 kDa

1 2 3 4 5 6 7 8 9

C

| pH | 7.4 | | 7.0 | | 6.6 | |
| TRAIL | - | + | - | + | - | + |

P-Akt   ◄ 60 kDa

Akt   ◄ 60 kDa

Actin   ◄ 43 kDa

1 2 3 4 5 6
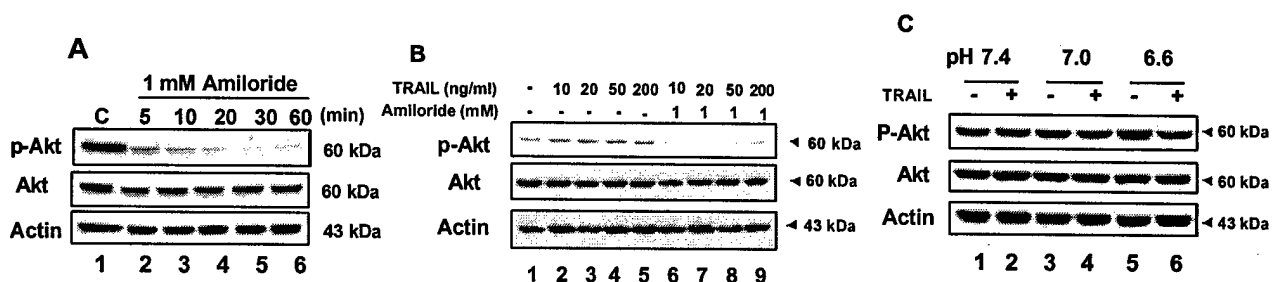
Figure 4. Effect of amiloride (A and B) or low extracellular pH (C) on the levels of phosphorylated Akt in DU-145 cells. (A) DU-145 cells were treated with 1 mM amiloride for various times (0- 60 min) and then harvested. (B) Cells were treated for 4 h with various concentrations of TRAIL (0-200 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. (C) Cells were treated with TRAIL (200 ng/ml) or without TRAIL (0 ng/ml) for 4 h at various extracellular pH (6.6-7.4) and then harvested. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody. Actin was shown as an internal standard.

To examine whether dephosphorylation of Akt during amiloride treatment is mediated through activation of phosphatase, we treated DU-145 cells with okadaic acid, a serine phosphatase inhibitor. Figure 5A shows that amiloride-induced Akt dephosphorylation was suppressed by pretreatment with 1 μM, but not by 0.1 μM, okadaic acid . It is well known that okadaic acid inhibits both protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), serine/threonine phosphatases, at higher concentrations (IC_{50} = 150 nM), but it inhibits only PP2A at low concentrations (IC_{50} < 0.1 nM).

These results confirmed that PP1 rather than PP2A plays an important role in the regulation of Akt phosphorylation, since recent studies have already demonstrated that dephosphorylation of Akt is regulated by PP1 (Xu et al., 2003). Unlike okadaic acid, sodium orthovanadate (Na$_3$VO$_4$), an inhibitor of tyrosin phosphatases, and emodin, a tyrosine kinase inhibitor, did not prevent dephosphorylation of Akt during treatment with amiloride (Fig. 5B). As a next step, we repeate d the procedure to examine a possible involvement of PP1 in the amiloride-induced dephosphorylation of Akt. Figure 6A shows that amiloride dephosphorylated (activated) PP1 and 1 μM okadaic acid substantially prevented dephosphorylation of PP1. Amiloride-promoted TRAIL-induced PARP cleavage was also significantly inhibited by treatment with 1 μM okadaic acid (Fig. 6B).

Figure 5. Effect of okadaic acid, sodium orthovanadate, or emodin on amiloride-induced dephosphorylation of Akt. (A) Cells were pretreated with okadaic acid (0.01-1 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (B) Cells were preteated with sodium orthovanadate (5-100 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (C) Cells were treated for 1 h with various concentrations of emodin (1-100 μg/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody. Actin was shown as an internal standard.
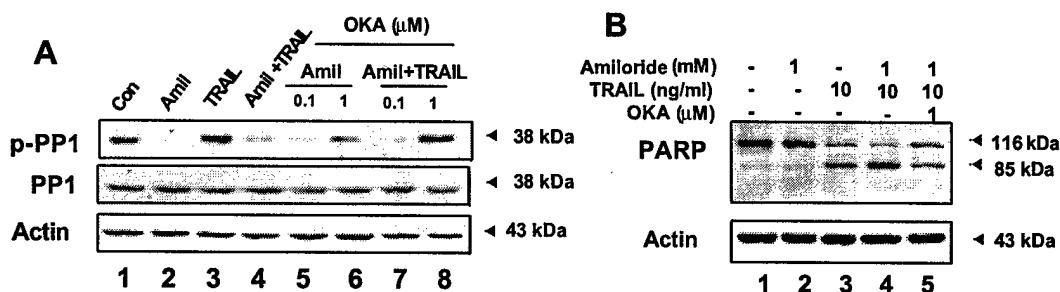

Figure 6. Effect of okadaic acid on amiloride-induced PP1 dephosphorylation (A) or TRAIL in combination with amiloride-induced PARP cleavage (B). (A) DU-145 cells were pretreated with okadaic acid (0.1-1 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (B) Cells were pretreated with 1 μM okadaic acid for 30 min and treated with TRAIL in the presence or absence of amiloride. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-PP1, anti-PP1, or anti-PARP antibody. Actin was shown as an internal standard.
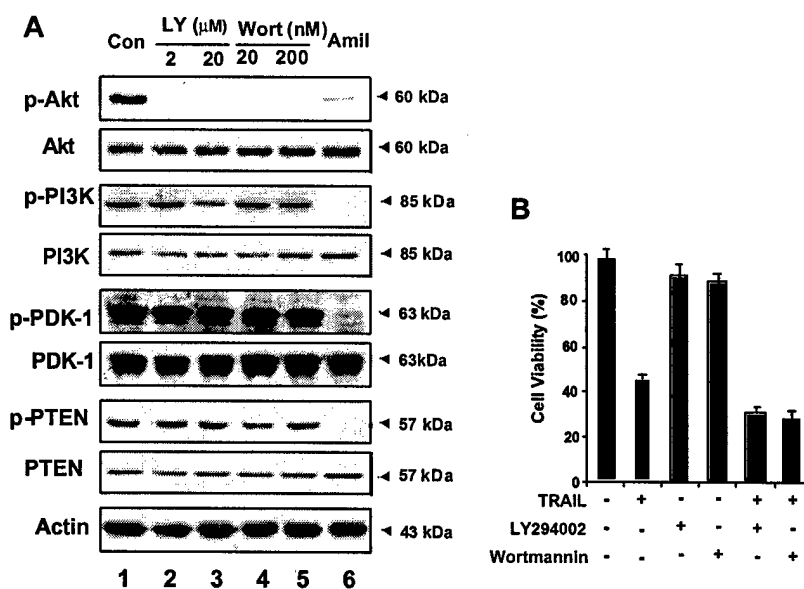
Effect of amiloride on kinases and phosphatases associated with the PI3K-Akt pathway

Previous studies demonstrated that Akt activation is also regulated through the PI3K-Akt pathway. We further examined whether amiloride specifically affects PP1 activity or nonspecifically affects the PI3K-Akt pathway-associated kinases and phosphatases. Figure 7A shows that amiloride dephosphorylated not only PP1 but also PTEN which is known to be a major negative regulator of the PI(3)K-Akt signaling pathway. Amiloride also dephosphorylated the PI3K-Akt pathway-associated kinases such as PI3K and PDK-1 (Fig. 7B). The effect of amiloride on Akt was compared with LY294002 and wortmannin, inhibitors of PI3K. Figure 8A shows that all these drugs dephosphorylated Akt. However, unlike amiloride, LY294002 and wortmannin did not dephosphorylate PI3K, PDK-1, and PTEN. Nevertheless, LY294002 and wortmannin promoted TRAIL-induced cytotoxicity (Fig. 8B). These results indicate that Akt inactivation (dephosphorylation) is responsible for enhancement of TRAIL cytotoxicity.
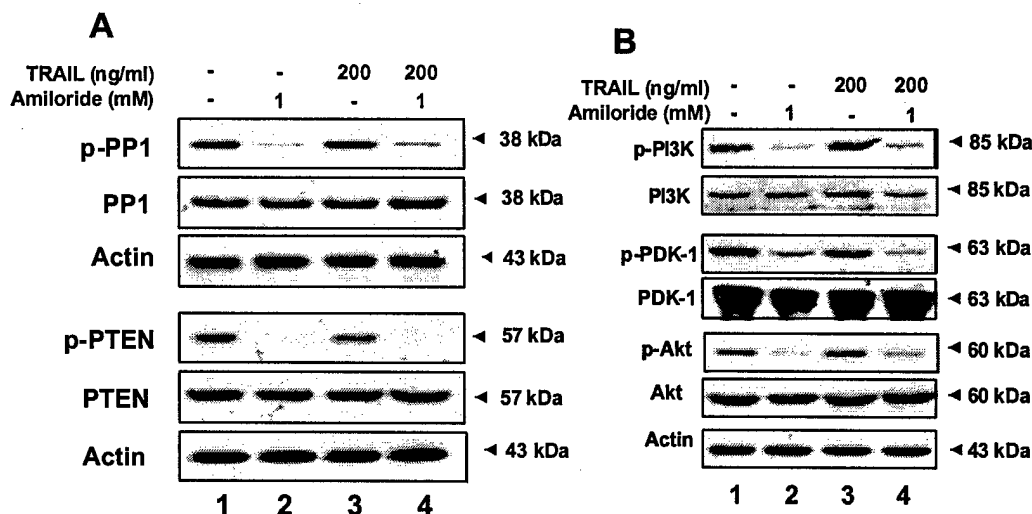
Figure 7. Effect of amiloride on phosphatases (A) or kinases (B) in the presence or absence of TRAIL. DU-145 cells were treated for 2 h with 1 mM amiloride in the presence or absence of 200 ng/ml TRAIL. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. Actin was shown as an internal standard.


Figure 8. Effect of LY294002 or wortmannin on kinases/phosphatases (A) and TRAIL-induced cytotoxicity (B) in DU-145 cells. (A) Cells were treated for 1 h with LY294002 (2-20 μM), wortmannin (20-200 nM), or 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. Actin was shown as an internal standard. (B) Cells were pretreated with 20 μM LY294002 or 200 nM wortmannin for 20 min and then treated or not treated with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments.

Mechanism of amiloride -induced alterations of phosphatase and kinase activities

Davis and Czech (44) reported that amiloride acts as an ATP analogue which causes the formation of nonproductive enzyme-substrate complexes. We hypothesized that amiloride competes with ATP and results in inhibiting the process of protein phosphorylation. To test this hypothesis, as a first step we investigated whether amiloride directly dephosphorylates active Akt. Figure 9 shows that amiloride did not directly dephosphorylate Akt when amiloride was mixed with phosphorylated Akt. In addition, amiloride did not inhibit dephosphorylation of Akt by PP1 (Fig. 9). As a

next step we examined whether amiloride blocks the kinase-mediated phosphorylation process. Figure 10A shows that unphosphorylated Akt was phosphorylated by active PDK-1 in vitro. Amiloride, but not DMSO, inhibited phosphorylation of Akt (Fig. 10B). Similar results were also observed in Figure 11. Casein kinase II (CK2), a protein kinase for PTEN, phosphorylated PTEN in vitro and its phosphorylation was blocked by amiloride (Fig. 11). These results suggest that amiloride blocks the kinase-mediated phosphorylation process by competing with ATP, but does not inhibit the phosphatase-mediated dephosphorylation.
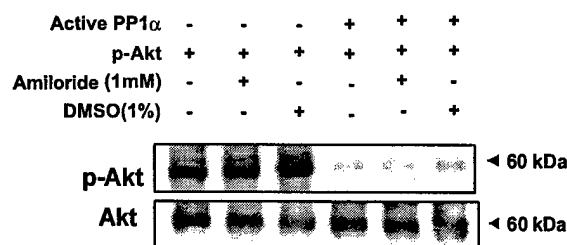
Figure 9. Effect of amiloride on phosphorylated Akt protein or PP1-mediated dephosphorylation of Akt. Purified active Akt protein was incubated with PP1, amiloride, or DMSO. The sample was separated by SDS-PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody.
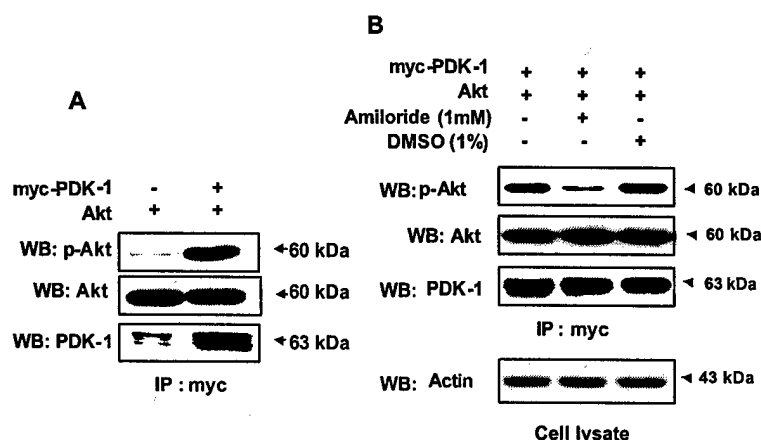
Figure 10. Effect of amiloride on PDK-1 kinase activity. DU-145 cells were transfected with pcDNA3myc-PDK-1. One day after transfection, myc-PDK-1 was immunoprecipitated with anti-myc antibody. For *in vitro* PDK-1 kinase assay, the immune complex was incubated with purified inactive Akt protein in the presence or absence of amiloride. The sample was separated by SDS-PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, or anti-PDK-1 antibody.
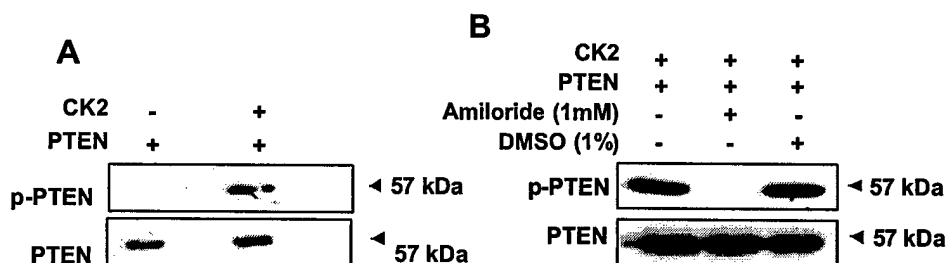
Figure 11. Effect of amiloride on CK2 kinase activity. For in vitro CK2 kinase assay, purified active PTEN protein was incubated with active CK2 in the presence or absence of amiloride. The sample was separated by SDS-PAGE and immunoblotted with anti-phospho-PTEN or anti-PTEN antibody.

Key research accomplishments:

We previously proposed that the PI3K-Akt signal plays an important role in TRAIL sensitivity. In this study we observed that amiloride enhances TRAIL-induced cytotoxicity in human prostate carcinoma DU-145 cell line. The mechanism of this enhancement is probably due to inhibition of PI3K and PDK-1 kinases as well as activation of PTEN and PP1 phosphatases by treatment with amiloride. Thus, our data support our proposed hypothesis.

Reportable Outcomes

Ki M. Kim and Yong J. Lee: Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the PI3K-Akt pathway. Manuscript in preparation.

Conclusions

Amiloride, an inhibitor of the PI3K-Akt signal transduction pathway, enhances TRAIL-induced cytotoxicity *in vitro*. Therefore, amiloride may also be a useful drug to promote TRAIL cytotoxicity in HER-2/neu overexpressing prostate tumor cells.

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